All Three LDL Receptor Homology Regions of the LDL Receptor-Related Protein Bind Multiple Ligands†

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ABSTRACT: The three complete human LDL receptor homology regions of the LDL receptor-related protein (sLRP2, sLRP3, and sLRP4) have been expressed in *Pichia pastoris* SMD1168 with constitutive coexpression of the receptor-associated protein (RAP). Each sLRP was purified to homogeneity after deglycosylation using a combination of anion-exchange and size exclusion chromatography. Mass spectrometry and N-terminal sequencing confirmed the identity of each fragment at purified yields of several milligrams per liter. Despite the large number of disulfide linkages and glycosylation sites in each LDL receptor homology region (sLRP), all were shown to be competent for binding to several LRP1 ligands. Each sLRP also bound human RAP, which is thought to be a generalized receptor antagonist, in solution-binding experiments. As expected, sLRP2 bound the receptor-binding domain of α_2-macroglobulin (residues 1304−1451). All three sLRPs bound human apolipoprotein-enriched β very low density lipoprotein, the canonical ligand for this receptor. All three sLRPs also bound lactoferrin and thrombin−protease nexin 1 complexes. Only sLRP4 bound thrombin−antithrombin III complexes. The results show that binding-competent LDL receptor homology regions (sLRPs) can be produced in high yield in *P. pastoris* and readily purified. Each sLRP has binding sites for multiple ligands, but not all ligand binding could be competed by RAP.

The LDL receptor-related protein, LRP1, plays a role in homeostasis of proteinases and proteinase inhibitors (*1*, *2*), in cellular signal transduction (*3*, *4*), and in vascular wall integrity and protection from atherosclerosis (*5*). LRP1 has also been implicated in the pathology of Alzheimer's disease (*6*). Proteins commonly found in senile plaques of Alzheimer's patient brains, including apolipoprotein E (apoE), α_2-macroglobulin (α_2M), lactoferrin, lipoprotein lipase, plasminogen activators and their proteinase complexes, and the receptor-associated protein (RAP), require LRP1 for internalization/clearance (*7*, *8*). Mature LRP1 is a two-chain molecule; the 515 kDa α-chain resides entirely in the extracellular space and is bound noncovalently to the 85 kDa β-chain (for reviews see refs *9* and *10*). The β-chain spans the membrane once and has been recently implicated in intracellular signaling (*3*, *4*). As with the LDL receptor, ligand binding is thought to occur within the complement-like domains in the α-chain (*11*).

Sequence alignments show that the α-chain contains three complete regions of similarity to the LDL receptor and another that is a partial repeat. These regions from human LRP1 have been expressed as both "minireceptors" and secreted fragments, and following on the nomenclature of Bu and Rennke, they are designated sLRP1 (the incomplete repeat containing cluster I), sLRP2 (contains cluster II), sLRP3 (contains cluster III), and sLRP4 (contains cluster IV) (Figure 1A) (*12*, *13*). The fact that RAP was required for proper folding suggested that it may be a folding chaperone for LRP1. In other experiments, it has been shown that RAP binds to all the subdomains except sLRP1 and competes for binding with nearly all LRP1 ligands (*14*). This result has been interpreted that RAP is a generalized antagonist for LRP1.

Using enzymatic and chemical cleavage of full-length LRP1, Herz was able to show that activated α_2M, uPA−PAI1 complexes, and RAP bound to a fragment of sLRP2 (*15*). Since this seminal report, many laboratories have demonstrated direct binding of various ligands to LRP1 and fragments of LRP1 either on cells or in fragments of LRP1 expressed on cells. For example, Bu and Rennke expressed sLRP2, sLRP3, and sLRP4 and were able to show RAP binding to all three (*13*). Horn et al. expressed small fragments of sLRP2 and used Biacore to demonstrate binding of RAP and tissue plasminogen activator−PAI1 complexes (*16*). Neels et al. also expressed sLRP2, sLRP3, and sLRP4 and carried out a panel of ligand-binding experiments but were unable to demonstrate binding of any ligands other than RAP to sLRP3. As the authors pointed out, this may have been due to the highly aggregated state of the recombinant proteins (*17*). Finally, fragments containing two

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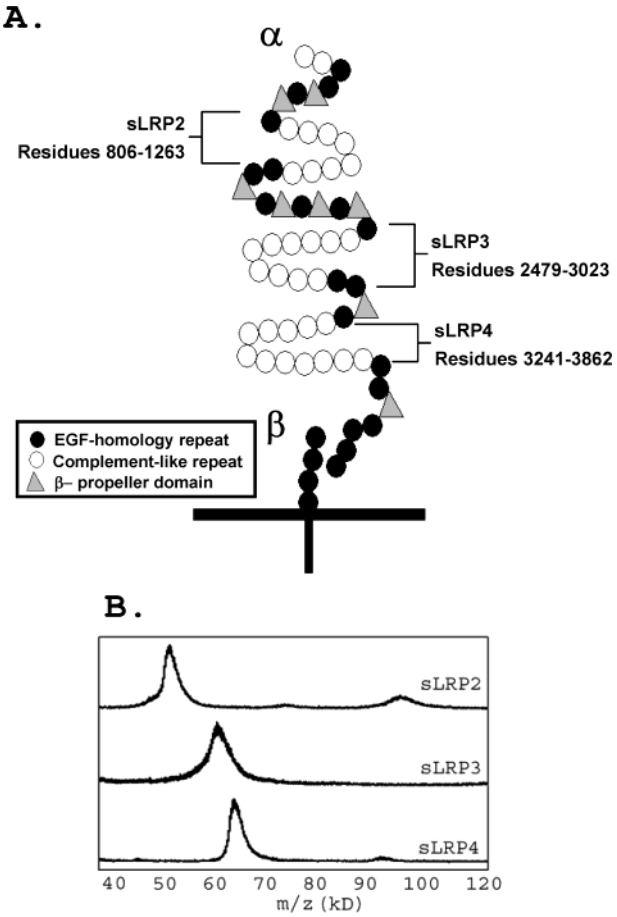

FIGURE 1: (A) Schematic diagram of LRP showing the EGF-like domains as filled circles, the complement-type repeats as open circles, and the propeller domains as gray triangles. The expressed LDL-like homology domains are shown in the bracketed regions, with their sLRP designations and corresponding amino acids. (B) MALDI-TOF mass spectrum of each sLRP in a sinapinic acid matrix. The masses obtained were sLRP2 = 50763 Da, sLRP3 = 61076 Da, and sLRP4 = 64047 Da.

ligand-binding domain segments have been expressed in *Escherichia coli* and shown to have weak binding affinity for α_2M (*18*).

With so many different ligands and so many different potential ligand-binding sites, we sought to extend the work of Bu and Rennke to express each complete LDL receptor homology region in a properly folded form in large quantities so that the ligand-binding domains could be purified and characterized in solution. We focused our ligand-binding studies on RAP, α_2M, apoE-enriched β-VLDL, lactoferrin, and two thrombin−serine protease inhibitor (SERPIN) complexes, thrombin−protease nexin 1 (Th−PN1) and thrombin−antithrombin III (Th−ATIII). The experiments presented here demonstrate that it is possible to obtain purified sLRPs in high yields by expression in *Pichia pastoris*. Purified, FLAG-tagged sLRPs were then used in solution-binding experiments to discover which sLRPs bind RAP, α_2M, apoE-enriched β-VLDL particles, lactoferrin, and thrombin−SERPIN complexes. All three sLRPs were competent to bind various ligands. Some of the binding results were as expected from previous results in the literature, while others were rather surprising. One surprise was the number of different ligands that bound to sLRP3. A second surprise was the not all ligands could be competed by RAP.

MATERIALS AND METHODS

Chemicals, Reagents, and Proteins. All chemicals and reagents were obtained from Fisher Scientific unless otherwise noted and were of the highest grade possible. Human thrombin and antithrombin III were obtained from Sigma Chemicals. Human protease nexin 1 (PN1) and the fusion protein of human RAP with glutathione *S*-transferase (GST−RAP) were prepared as described previously (*15*, *19*). Human lactoferrin was a generous gift from Agennix, and apoE-enriched β-VLDL was from Intracel. 1,3,4,5-Tetrachloro-3α,3α-diphenylglycouril (Iodogen) was from Pierce Chemicals. ^{125}INa was purchased from Amersham. Human thrombin was radioiodinated using Iodogen as previously described (*20*). Specific activities ranged from 15000 to 20000 cpm/ng. Confirmation of GST−RAP production was obtained by western blotting using polyclonal rabbit anti-GST antisera (graciously donated by Dr. Peter van der Geer). The protein concentration was determined by BCA protein assay (Pierce Chemicals), and fractions were stored at −70 °C. Full-length human α_2M was obtained from Sigma Chemicals and activated as previously described (*21*). The receptor-binding domain (RBD, residues 1304−1451) of human α_2M was expressed in *E. coli* and refolded overnight on the nickel column as previously described (*22*). After refolding, the α_2M(1304−1451) was eluted from the nickel column with 20 mM EDTA in 50 mM Tris and 500 mM NaCl, pH 8.0, and dialyzed into Tris-buffered saline, pH 7.5, also containing 300 mM NaCl.

Coexpression of LRP1 Ligand-Binding Domains and RAP in P. pastoris Strain SMD1168. Production of properly folded soluble ligand-binding domains of LRP1 requires coexpression of RAP as demonstrated by Bu and Rennke (*13*). Therefore, we first tranformed the protease-deficient strain of *P. Pastoris*, SMD1168, so that it would constituitively express RAP under the control of the GAP (glyceraldehyde-3-phosphate dehydrogenase) promoter. Primers engineered to PCR amplify the rat RAP gene as well as to incorporate a 5′ *Xho*I exonuclease site (CTCGAG) and *Kex*II protease site (AAAGA) and a 3′ *Not*I exonuclease site (GCGGCCGC) were 5′-CGTACGTACTCGAGAAAAGATACTCGCGG-GAGAAGAATG and 3′-GGCCGGGCGCGGCCGCTCA-GAGCTCATTGTGCCG. The PCR-amplified gene was subcloned into a pGAPzα vector (Invitrogen, Inc.) using the *Xho*I and *Not*I cloning sites. Approximately 15 μg of maxiprepped pGAPzα-RAP plasmid was digested with *Bgl*II for 1 h at 37 °C, purified by Gene-Clean (Bio-101, Inc.), and added to electroporated SMD1168 *P. pastoris* cells prepared according to Invitrogen protocols. Cells were plated on YPD plates containg 100 mg/mL zeocin. Confirmation of RAP expression was obtained by western blots using a rabbit polyclonal antibody against RAP. The RAP gene was subcloned behind the α-factor leader sequence for secretion, but it was found to be retained in the cells presumably because the ER localization signal was functional (*23*).

The full-length cDNA for human LRP1 was generously donated by Dr. Joachim Herz (*24*), and the same sLRP fragments described by Bu and Rennke (Figure 1A) were constructed. All 5′ primers were made to include a *Xho*I endonuclease site and *Kex*II protease cleavage site, while 3′ primers were made to include a *Not*I endonuclease site. Constructs were also made that included a FLAG anibody

epitope tag at the C-terminus, and since these are the constructs used here, the FLAG-containing primers are given: sLRP2, 5′-CGTACGTACTCGAGAAAAGAAAATGCCGGGTGAACAATGG; sLRP2, 3′-CGTACGTAGCGGCCGCTCACTTGTCGTCGTCGTCCTTGTAGTCGCTGCGGCAGCTCTCGCC; sLRP3, 5′-CGTACGTACTCGAGAAAAGACTCTCTCCATGCCGAATC; sLRP3, 3′-CGTACGTAGCGGCCGCTCACTTGTCGTCGTCGTCCTTGTAGTCAGCCTTGCAGCTGTGGGG; sLRP4, 5′-CGTACGTACTCGAGAAAAGAAATCACCCCTGCAAGGTC; sLRP4, 3′-CGTACGTAGCGGCCGCTCACTTGTCGTCGTCGTCCTTGTAGTCGGCCTTGCACGTGTTGTGCGTC.

Each sLRP fragment was PCR amplified and subcloned into the pPIC9 and then into the pPIC9K vector (Invitrogen, San Diego, CA) as described previously (*25*). Each sLRP-containing pPIC9K vector was used to tranform the RAP−SMD1168 strain by spheroplast transformation as described previously (*25*). The pooled transformants were reselected for zeocin, and G418$^+$-resistant colonies were selected by replica plating. Approximately eight G418 colonies of each sLRP construct were tested for expression of both the sLRP and RAP by western blotting using either the anti-RAP antibody or a polyclonal antibody against full-length LRP1 generously provided by Dr. Bu (*13*).

Expression of sLRPs. P. pastoris strains producing each sLRP (sLRP2, sLRP3, or sLRP4) were stored at −70 °C in glycerol stocks, and aliquots were removed as needed to inoculate 5 mL of 1% BMGY (*25*) and grown for 24 h at 30 °C. These cultures were then used to inoculate 1 L cultures of 1% BMGY in baffled 4 L shake flasks which were grown with vigorous shaking with cheesecloth covers for 48 h to an OD_{600} of approximately 60. The yeast were collected by centrifugation at 3500*g* and resuspended in 500 mL of 1% BMMY in the same flasks. After 24 h, the supernatant containing the secreted sLRP was collected by centrifugation at 5000*g* and stored at −70 °C.

Purification of sLRP Fragments. Culture supernatants (typically 1 L volume) were thawed at 37 °C, and precipitate was removed by vacuum filtration through a 0.8 μm filter, followed by a 0.22 μm filter. Supernatants were diluted with 2 mM $CaCl_2$ to a conductivity between 9 and 13.0 mΩ and loaded at 4 °C onto an FPLC Hi Load Q 26/10 anion-exchange column (Amersham/Pharamacia, Inc.) at a flow rate of 6.0 mL/min. After being washed with buffer A (50 mM MES, 2 mM $CaCl_2$, pH 6.5), the protein was eluted with a step gradient (25%, 50%, 100%) with buffer B (50 mM MES, 2 mM $CaCl_2$, 1 M NaCl, pH 6.5). Western blot analysis of eluted fractions confirmed the presence of each sLRP in the 50% fraction. The 50% fraction was then concentrated using Centriprep/Centricon 30 (Millipore, Inc.) to a final concentration of 10 mg/mL as determined by BCA protein assay (Pierce Chemicals).

Final purification of each sLRP was facilitated by deglycosylation with endoglycosidase H (EndoH) (Sigma Chemical Co., St. Louis, MO). Approximately 1 mg of each sLRP was deglycosylated using 0.01 unit of endoglycosidase H in 10 mM sodium acetate buffer, pH 5.5, at 37 °C for 6.5 h. Deglycosylated sLRPs were finally purified by Superdex 200 16/60 size exclusion FPLC chromatography (Amersham/Pharamacia, Inc.) in MB150 (10 mM HEPES, 2.5 mM $CaCl_2$, 150 mM NaCl, 1 mM $MgCl_2$, pH 7.5). Each sLRP eluted at the calculated monomeric molecular mass and was stored in aliquots at −70 °C. Each sLRP was characterized by SDS−PAGE and was shown to react with the polyclonal antibody against full-length LRP1. Although this antibody reacts weakly with sLRP3, the reaction was strong enough to use as an indicator of expression yields and to follow the protein through purification. Polyclonal antibodies were raised in rabbits against each purified sLRP by Chemicon Inc. and used to probe western blots for the presence of each sLRP.

MALDI-TOF MS Analysis of Purified sLRP Fragments. Samples (0.5 ng) of each sLRP were acidified with 1% trifluoroacetic acid, mixed 1:5 with sinapinic acid matrix solution [10 mg/mL of sinapinic (3,5-dimethoxy-4-hydroxycinnamic acid, 224 g/mol) dissolved in 3 parts CH_3CN to 7 parts 2% TFA], and analyzed by MALDI-TOF on a PE Biosystems DE-STR MALDI-TOF mass spectrometer calibrated with enolase/enolase dimer mass standards.

sLRP Interaction with GST−RAP. The GST fusion protein of human RAP (GST−RAP) was purified according to the methods of Orlando et al. (*26*). Complexes of each sLRP with GST−RAP were formed by mixing 3 μg (0.05 nmol) of GST−RAP and 50 μg of each sLRP fragment (approximately 1 nmol) at 37 °C for 2 h. Control experiments were performed side by side with equimolar amounts of GST in place of GST−RAP. Complexes of each sLRP with GST−RAP were captured with the addition of 10 μL of anti-GST agarose prepared by coupling polyclonal anti-GST antibody (Pharamacia) to protein A−agarose and cross-linking with dimethyl pimelimidate (Pierce Chemicals). After the anti-GST agarose was mixed with each complex and incubated at room temperature for 30 min, the agarose was recovered by centrifugation, washed three times in MB150 with 0.1% Tween-20, resuspended in 10 μL of 2× SDS reducing sample buffer, electrophoresed on a 10% SDS−PAGE gel, and transferred to nitrocelluose. Captured sLRPs were identified by western blotting and detected with polyclonal antisera raised against the specific sLRP being detected. Experiments were carried out either in MB150, which contains 2.5 mM $CaCl_2$, or in the same buffer without calcium, and instead containing 5 mM EDTA.

a_2-Macroglobulin Binding Assays. Purified α_2M(1304−1451) was incubated with 0.1 nmol of each sLRP at 37 °C for 2 h. GST−RAP competition was ascertained by addition of a 50-fold molar excess (300 μg) to the sLRPs 30 min prior to addition of the α_2M(1304−1451). Subsequently, 80 μL of antiFLAG M2 agarose bead 50% slurry was added, and incubation was continued for 30 min at 25 °C. Prior to incubation, the anti-FLAG M2 agarose was blocked by washing three times in MB150 plus 3% BSA. Each agarose sample was centrifuged for 2 min, washed three times with MB300 with 0.1% Tween-20, resuspended in 2× reducing SDS sample buffer, boiled for 10 min at 100 °C, loaded onto a 15% SDS−PAGE gel, electrophoresed, and transferred to nitrocellulose. Western blotting was accomplished using a polyclonal anti-α_2M antibody (Sigma Chemicals). Similar experiments were also performed with full-length α_2M that was either not activated, activated according to published protocols (*21*), or activated and then cleaved with papain. The results from these experiments showed that full-length α_2M bound nonspecifically to the FLAG M2 resin, and binding to sLRPs was not reproducibly over background.

Table 1: Characterization of Purified sLRPs

	N-terminal sequence[a]		molecular mass (Da)[b]	
sLRP	expected	observed	expected	observed
2	YVPPPQCQPGEFACA	YVPPPQ−QPGEFA−A	50516	50763
3	LSPCRINNGGCQDLC	LSP−RINNGG−QDL−	60932	61076
4	NHPCKVNNGGCSNLC	NHP−KVNNGG−SNL−	63770	64047

[a] The cysteines are in the oxidized form and therefore show up as blank cycles (−) in Edman degradation. [b] The expected molecular mass was calculated from the amino acid sequence, taking into account the fact that the cysteines are in the oxidized form and there is an *N*-acetylglucosamine on each consensus asparagine.

Specific binding to the receptor-binding domain that results from papain cleavage was observed.

Lactoferrin Binding Assays. Human lactoferrin (Agennix) was mixed in a 1:1 molar ratio with each FLAG-tagged sLRP in MB150 and incubated for 2 h at 37 °C. GST−RAP competition was ascertained by addition of a 50-fold molar excess (300 μg) to the sLRPs 30 min prior to addition of the lactoferrin. The sLRP−lactoferrin complexes were captured by addition of 100 μL of a 50% slurry of blocked anti-FLAG M2 agarose for 30 min at 25 °C in order to capture the FLAG-tagged sLRPs. Each agarose sample was centrifuged for 2 min, washed three times with MB150 with 0.1% Tween-20, resuspended in 2× reducing SDS sample buffer, boiled for 10 min at 100 °C, loaded onto a 10% SDS−PAGE gel, and electrophoresed. The lactoferrin was detected by western blot using a polyclonal anti-lactoferrin antibody (Sigma Chemicals).

ApoE-Enriched β-VLDL Binding Assays. ApoE-enriched β-VLDL particles (7.5 μg) were mixed with 5 μg of each sLRP−FLAG fragment at 37 °C for 2 h. GST−RAP competition was ascertained by addition of a 50-fold molar excess (300 μg) to the sLRPs 30 min prior to addition of the apoE-enriched β-VLDL particles. Competition for binding by a polyclonal antibody against apoE (Chemicon, Inc.) and by lactoferrin was each carried out in a fashion similar to the RAP competition experiments. Following incubation, each mixture was captured with anti-FLAG M2 agarose as already described, loaded onto a 13% SDS−PAGE gel, electrophoresed, and transferred to nitrocellulose. Western blotting of the apoE contained in the β-VLDL particles was accomplished using a commercially available polyclonal antibody to apoE.

Assays for Binding of Thrombin−SERPIN Complexes. ^{125}I-Labeled thrombin−SERPIN complexes (500 ng) were formed at 37 °C for 30 min in MB150BSA (1% BSA). The degree of complex formation was ascertained by 13% SDS−PAGE in which ^{125}I-thrombin−SERPIN complexes and ^{125}I-thrombin alone were separated and quantitated by phosphorimaging (*19*, *27*). Thrombin−SERPIN complexes (62.5 nM in 20 μL) were mixed with equimolar amounts of each sLRP and incubated at 37 °C for 1 h. Each sLRP was also preincubated with 5 μL of a 2.5 μM solution of RAP for 30 min and then mixed with the thrombin−SERPIN complexes for 1 h. Following incubation, each mixture was incubated with 50 μL of a 50% slurry of anti-FLAG M2 agarose for 30 min at 25 °C as already described, loaded onto a 13% SDS−PAGE gel, and electrophoresed. Gels were fixed for 30 min in 5% ethanol and 7% acetic acid and imaged for 12 h on a Bio-Rad molecular imager.

RESULTS

Expression of Soluble LRP1 Ligand-Binding Domains in P. pastoris. The three LDL receptor homology regions of the human LDL receptor-related protein (sLRP2, sLRP3, and sLRP4) that were previously described by Bu and Rennke (Figure 1A) have been expressed in *P. pastoris* SMD1168 with coexpression of the receptor-associated protein (RAP). The sLRPs contain all of the ligand-binding complement-type repeat clusters (CLII in sLRP2, CLIII in sLRP3, and CLIV in sLRP4) bracketed by EGF-like domains, but they do not contain the β-propeller domains. The *P. pastoris* strain used to express the sLRPs expressed rat RAP constitutively behind the glyceraldehyde-3-phosphate dehydrogenase promoter. As was the case for expression of RAP in mammalian cells (*13*), the endoplasmic reticulum localization signal functioned in the *P. pastoris* cells to retain the RAP protein within the cell (data not shown). Each sLRP ligand-binding domain was produced by methanol induction of the alcohol oxidase promoter. Experiments were not performed to assess expression levels in the absence of coexpression of RAP; however, experiments in which the glycerol was depleted indicated that reduction of RAP expression resulted in a concomitant reduction in production of sLRP2 (data not shown).

Purification and Characterization of the Soluble LRP1 Ligand-Binding Domains. Each sLRP was produced in shake flasks with purified yields ranging from 2 to 10 mg/L using previously described methods (*25*). Initial purification was accomplished using anion-exchange chromatography on HiLoad Q FPLC. To obtain fully purified protein, it was necessary to remove the oligosaccharides using endoglycosidase H, after which the proteins could be finally purified by size exclusion chromatography. Endoglycosidase H leaves behind one *N*-acetylglucosamine (GlcNAc) residue at each N-linked sugar attachment site, and there are five of these on sLRP2, six of these on sLRP3, and three of these on sLRP4. MALDI-TOF mass spectrometry showed the presence of pure protein at the expected molecular mass (including one GlcNAc per asparagine attachment site) for each sLRP. N-Terminal sequencing confirmed the purity and identity of each fragment (Figure 1B, Table 1). Size exclusion chromatography of the purified, deglycosylated protein showed that each eluted at its expected monomeric molecular mass, suggesting that proper folding of each sLRP was accomplished by the expression system. Comparison of the molecular masses determined by mass spectrometry with the apparent molecular masses on SDS−PAGE showed that the reduced proteins migrate at a slightly higher than expected apparent molecular mass on SDS−PAGE after deglycosylation. This is consistent with previous observations for purified LDL receptor, which has a molecular mass of ~115

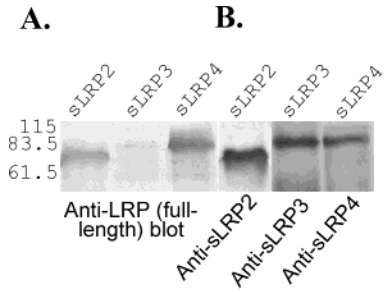

FIGURE 2: Western blot analysis of purified sLRPs. Reducing 10% SDS−PAGE analysis of each sLRP (2 μg) was performed, and the proteins were transferred to nitrocellulose. (A) Each sLRP was visualized with a polyclonal antibody against full-length LRP. (B) Each sLRP was visualized with a polyclonal antibody raised against each purified sLRP.

kDa (*28*, *29*) but migrates at an apparent molecular mass of 153 kDa even after deglycosylation (*30*). The oxidized proteins, due their highly disulfide-bonded structure, migrate in a broad band and at lower than expected molecular masses (data not shown).

Interaction of the Soluble LRP1 Ligand-Binding Domains with Antibodies and RAP. Each pure protein reacted with the polyclonal antibody raised to the full-length naturally occurring protein (a generous gift of Dr. Bu) (Figure 2A). The binding of sLRP3 to this polyclonal antibody appeared weaker than binding to sLRP2 or sLRP4. Polyclonal antibodies were raised against each purified sLRP fragment, and each antiserum reacted most strongly with the sLRP fragment to which it was raised (Figure 2B). Experiments to test the cross-reactivity of the polyclonal antibodies raised against each sLRP showed that sLRP2 did not cross-react with antibodies raised against sLRP3 or sLRP4. On the other hand, sLRP3 cross-reacted with antibodies against sLRP2 and weakly with antibodies against sLRP4. Finally, sLRP4 cross-reacted with antibodies against sLRP2 but not with antibodies against sLRP3 (data not shown).

RAP has been reported to be a generalized antagonist of LRP1 and has been shown to bind at multiple sites in various expressed LRP1 fragments (*13*, *15*, *18*). To determine whether RAP binds to each of the purified sLRPs, solution binding experiments were performed. In these experiments, each purified sLRP was incubated with the GST fusion of human RAP in solution, and the complex was then pulled down using anti-GST agarose beads. The LRP1 fragments bound avidly to glutathione beads by virtue of cross-disulfide bond formation, and therefore anti-GST antibody beads were used in all GST pull-down experiments. GST−RAP bound to all three sLRPs (Figure 3A−C). No binding of sLRPs to RAP was observed when the pulldowns were done in EDTA (Figure 3D,E).

Ligand-Binding Experiments. Solution binding assays were used to determine whether various ligands bound to each sLRP. For these experiments, each C-terminally FLAG-tagged sLRP was incubated with each ligand in solution prior to FLAG capture by monoclonal anti-FLAG M2 antibody agarose beads. The proteins bound to the beads were then electrophoresed, and each ligand was detected with an appropriate antibody.

Full-length activated α_2M bound nonspecifically to all fragments and to the control antibody beads (data not shown). Nonspecific binding was observed with both activated and

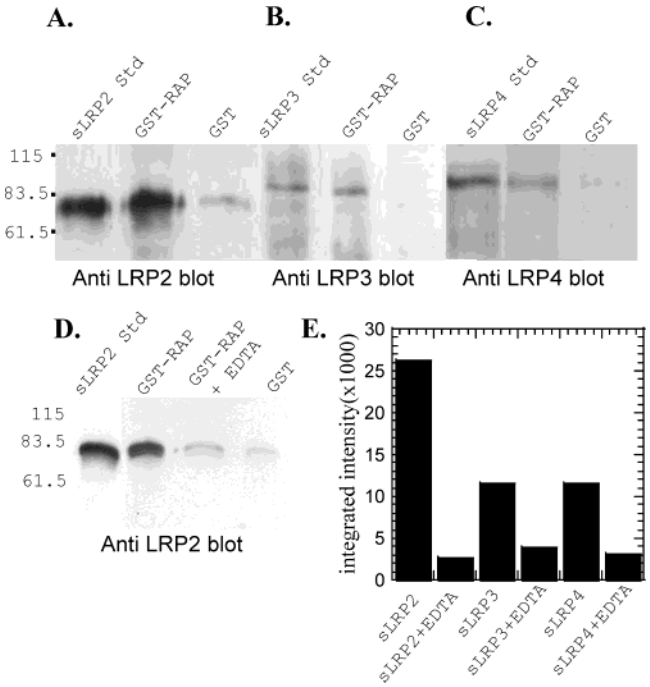

FIGURE 3: Pull-down experiment in which each purified sLRP (50 μg) was captured with GST−RAP (3 μg). Control experiments using only GST were also performed. Each sLRP/GST−RAP complex was pulled down using protein A beads to which was covalently linked anti-GST antibody (Pharmacia). Captured sLRPs were separated on reducing 10% SDS−PAGE gels, transferred to nitrocellulose, and visualized using the antibody specific for the sLRP that was captured. (A) Capture of sLRP2. (B) Capture of sLRP3. (C) Capture of sLRP4. (D) Representative GST−RAP pull down of sLRP2 in the presence and absence of EDTA. (E) Bar graph of the integrated intensity of each protein band for each sLRP pulled down with GST−RAP in the presence and absence of EDTA.

nonactivated full-length α_2M (*21*). The activated α_2M was cleaved with papain as described by Sottrup-Jensen et al., and the mixture of cleaved products was also used in sLRP binding experiments. These showed specific binding of sLRP2 to the receptor-binding domain fragment (data not shown). We therefore prepared the receptor-binding domain [α_2M(1304−1451)] by expression in *E. coli* as described previously (*22*). Figure 4A shows that the binding of the α_2M(1304−1451) was completely inhibited by RAP as was expected (*15*, *31*). As expected from previous results, binding of the α_2M(1304−1451) was strongest to sLRP2, no binding was observed to sLRP3 or sLRP4, and binding was inhibited by EDTA (Figure 4B) (*18*, *32*, *33*).

Binding of the canonical ligand, apoE-enriched β-VLDL particles, was observed to each of the sLRPs. ApoE-enriched β-VLDL particles bound to an equivalent extent to each of the three sLRPs. RAP did not compete for binding even at an 80-fold molar excess (Figure 5A). Experiments were also performed to ascertain whether the binding of apoE-enriched β-VLDL particles could be diminished in the presence of EDTA. As was originally reported by Beisiegel et al., binding of apoE-enriched β-VLDL particles was similar in the presence and absence of EDTA (*34*). Because the binding of apoE-enriched β-VLDL particles could not be competed by RAP, specificity of binding was demonstrated by competition with a polyclonal antibody against apoE (Figure 5C).

Lactoferrin has also been shown previously to be a bona fide ligand of LRP1 (*35*). Lactoferrin bound to all three

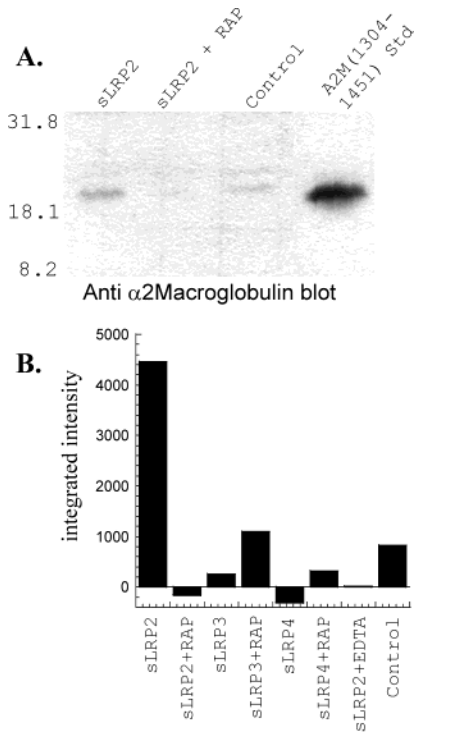

FIGURE 4: Pull-down experiment in which purified α_2M(1304−1451) was captured using each sLRP. α_2M(1304−1451)/sLRP−FLAG complexes were captured using anti-FLAG M2 agarose. Control experiments in which no sLRP−FLAG was added were used to detect nonspecific binding of α_2M(1304−1451) to the anti-FLAG agarose beads. In each case, a competition with GST−RAP was also performed. Captured α_2M(1304−1451)/sLRP2−FLAG complexes were separated on 15% SDS−PAGE, and α_2M(1304−1451) was visualized using a polyclonal antibody raised to full-length α_2M. (A) α_2M(1304−1451) bound to sLRP2−FLAG, and binding was competed by RAP. (B) Bar graph of the integrated intensity of captured α_2M(1304−1451) protein bands in the presence and absence of GST−RAP and showing that no binding was detected to sLRP3−FLAG or sLRP4−FLAG or to sLRP2−FLAG in the presence of EDTA.

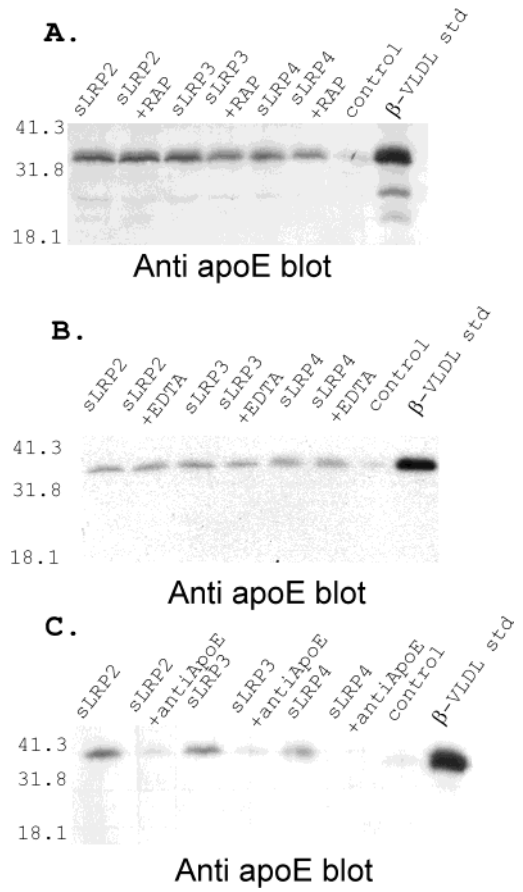

FIGURE 5: Pull-down experiment in which apoE-enriched β-VLDL binding to each sLRP was studied. Control experiments in which no sLRP−FLAG was added were used to detect nonspecific binding of apoE-enriched β-VLDL to the anti-FLAG agarose beads. In each case, a competition with GST−RAP and a polyclonal antibody raised to apoE was also performed. Experiments were also conducted in the presence and absence of EDTA. ApoE/sLRP−FLAG complexes were captured using anti-FLAG M2 agarose. Captured complexes were separated on 13% SDS−PAGE and transferred to nitrocelluose, and apoE was visualized using a polyclonal antibody raised to full-length apoE. (A) Capture of apoE in the presence and absence of GST−RAP. (B) Capture of apoE in the presence and absence of EDTA. (C) Capture of apoE in the presence and absence of a polyclonal antibody raised to apoE.

sLRPs, but the strongest binding appeared to be to sLRP2. As with the apoE-enriched β-VLDL particles, the binding of lactoferrin was not inhibited by even large excesses of RAP (Figure 6A). Binding was also not inhibited by addition of EDTA (Figure 6B). As has been reported previously (*35*), lactoferrin binding inhibited the binding of apoE-enriched β-VLDL particles (Figure 6C).

Binding to each of the sLRPs was also measured for thrombin−SERPIN complexes in solution. Thrombin−protease nexin 1 complexes (covalent complexes migrate at 80 kDa) bound to all three sLRPs, and RAP competed for binding of sLRP3 and sLRP4 but not of sLRP2 (Figure 7A,C). Thrombin−ATIII complexes (covalent complexes, 95 kDa) bound mainly to sLRP4, and binding was efficiently competed by RAP (Figure 7B,D).

DISCUSSION

P. pastoris expression of each sLRP with the coexpression of RAP is an effective way of obtaining large quantities of soluble LRP1 extracellular domain fragments. To our knowledge, this report represents the first demonstration, including N-terminal sequencing and MALDI-TOF mass spectrometry, of production of large quantities of purified monomeric sLRP fragments for ligand-binding studies. The availability of purified receptor fragments and specific antibodies has allowed us to begin to rigorously define the different binding sites of a few of the many important ligands of LRP1.

All three sLRPs bind RAP. Many other groups have previously shown that LRP1 binds RAP, but this is the first demonstration that the EndoH-treated, purified sLRPs bind RAP. Thus, the RAP interaction is a protein−protein interaction that does not seem to be dependent upon glycosylation. It is also important to note that the sLRPs studied here contain only the EGF-like domains and ligand binding or complement-type repeats and not the β-propeller domains of LRP1. The results therefore suggest that RAP binding does not require the β-propeller domains of LRP1.

The receptor-binding domain of α_2M bound preferentially to sLRP2, and binding was inhibited by RAP and by EDTA as has been previously reported (*15*, *18*, *31*−*33*). Although others have reported binding to sLRP4, we were unable to observe specific binding of the receptor-binding domain of α_2M to sLRP4 (*17*).

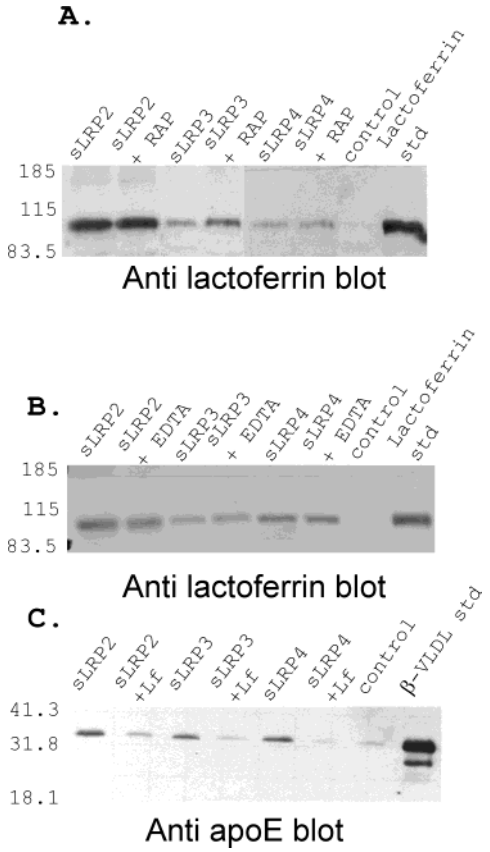

FIGURE 6: Pull-down experiment in which human lactoferrin binding to each sLRP was studied. Control experiments in which no sLRP−FLAG was added were used to detect nonspecific binding of lactoferrin to the anti-FLAG M2 agarose beads. In each case, a competition with GST−RAP was also performed. Experiments were also conducted in the presence and absence of EDTA. Lactoferrin/sLRP−FLAG complexes were captured using anti-FLAG M2 agarose, separated on 10% SDS−PAGE, and transferred to nitrocelluose, and lactoferrin was visualized using a polyclonal antibody raised to full-length lactoferrin. (A) Capture of lactoferrin in the presence and absence of GST−RAP. (B) Capture of lactoferrin in the presence and absence of EDTA. (C) Competition experiment in which apoE-enriched β-VLDL was captured in the presence and absence of lactoferrin.

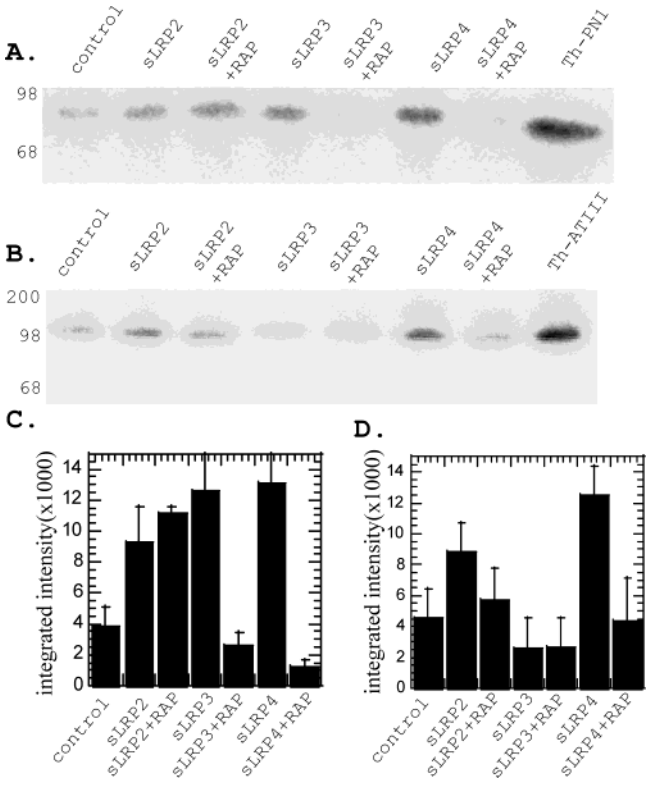

FIGURE 7: ^{125}I-Thrombin−PN1 complexes were formed and mixed with simultaneous additions of FLAG-tagged sLRPs. Control experiments in which no sLRP was added were used to detect nonspecific binding of the complexes to the anti-FLAG agarose beads. ^{125}I-Thrombin−PN1/sLRP complexes were captured using anti-FLAG M2 agarose and electrophoresed on a 13% SDS−PAGE gel, and ^{125}I-labeled thrombin complexes were detected for 12 h on a Bio-Rad phosphoimager. (A) Capture of ^{125}I-thrombin−PN1 complexes by each individual sLRP−FLAG with or without competition with RAP. (B) The same capture experiment as described in (A) was performed except that the FLAG-tagged sLRPs were incubated with ^{125}I-thrombin−ATIII complexes in the presence or absence of RAP. (C) Bar graph depicting the average integrated intensity of captured ^{125}I-thrombin−PN1 with each sLRP in the presence and absence of GST−RAP. Error bars were calculated on the basis of two independent experiments. (D) Bar graph depicting the average integrated intensity of captured ^{125}I-thrombin−ATIII complexes in the presence and absence of GST−RAP. Error bars were calculated on the basis of two independent experiments.

All three sLRPs also bound the well-established LRP1 ligands, apoE-enriched β-VLDL particles, and lactoferrin. This is the first demonstration of binding of bona fide LRP1 ligands to the sLRP3 region. Previous studies had only been able to show binding of RAP to this region (*17*). Our results show convincingly that sLRP3 binds to apoE-enriched β-VLDL particles, lactoferrin, and thrombin−PN1 complexes. Although the pull-down binding assays are qualitative, we used a 1:1 molar ratio of sLRP to ligand, and binding was equivalent to sLRP2, sLRP3, and sLRP4 for apoE-enriched β-VLDL particles and thrombin−PN1 complexes, although lactoferrin appeared to bind more strongly to sLRP2.

Although RAP competed for binding of α_2M, binding of apoE-enriched β-VLDL particles was not sensitive to competition by even large excesses of RAP. RAP competes for cellular internalization/degradation of most LRP1 ligands and is thought to be a generalized antagonist of ligand binding to LRP1 (*36*−*40*). Our results are consistent with other reports in which RAP competed for binding of ligands such as α_2M to cell surface LRP1 but RAP did not compete for cell surface binding of apoE-enriched β-VLDL particles (*41*). Our results appear to be inconsistent with the observation that RAP can block binding of apoE-enriched β-VLDL particles in ligand-blotting experiments. In these experiments, full-length LRP1 was electrophoresed and blotted and then incubated with RAP. The blot was afterward probed for binding of apoE-enriched β-VLDL particles (*15*). It is possible that RAP, being a highly charged molecule, somehow is able to compete for binding to LRP1 in the solid phase of a ligand blot but is not able to effectively compete in solution. In further experiments, Ziere et al. also showed that RAP did not inhibit lactoferrin binding to cells (*42*). This group concluded that the lactoferrin was probably binding to a different receptor, and not LRP1, but our results show definitively that lactoferrin binds to LRP1; the binding is just not inhibited by RAP. Other recent experiments show only a partial inhibition of lactoferrin binding to LRP1 on cells (*43*). Thus, RAP may not be a completely general antagonist for LRP1 ligand binding.

The two ligands that could not be competed by RAP also bound in the presence of EDTA. Although binding of RAP to each of the sLRPs was inhibited by EDTA, the binding of apoE-enriched β-VLDL particles to each of the three sLRP fragments was the same in the presence and absence of EDTA. We are not the first to report that the binding of LRP1 to β-VLDL particles appears unaffected by EDTA (*34*, *44*). Beisiegel et al. showed by cross-linking of apoE to cell surface proteins that binding to LRP1, identified by western blot, was similar in the presence and absence of EDTA while binding to the LDL receptor was abolished. Others have shown that, on cells, there are multiple binding sites for β-VLDL particles, and depending on cell type, a portion of the binding remains in the presence of EDTA. Our results also showed that lactoferrin binding was unaffected by EDTA. These are the two ligands that are not sensitive to RAP inhibition. It is possible, therefore, that the RAP binding site is distinct from the apoE and lactoferrin binding sites and that the RAP binding site somehow involves calcium while the apoE and lactoferrin binding sites do not. Calcium has been shown to be required for proper folding of ligand-binding domains of the LDL receptor, and this is most likely true for LRP as well (*45*). Experiments in which LRP was electrophoresed in SDS required the addition of calcium before apoE could bind, as assessed by ligand blotting (*46*). Thus, the most likely explanation of our results is that calcium is required for proper folding of LRP1 but not for apoE binding.

Thrombin−SERPIN complex binding to the sLRPs revealed that thrombin−ATIII complexes bound primarily to sLRP4 while thrombin−PN1 complexes bound to sLRP2, sLRP3, and sLRP4. RAP competed for thrombin−ATIII binding to sLRP4, and this is consistent with results from cell-binding experiments reported by Strickland's group, which showed that RAP completely inhibits thrombin−ATIII binding to LRP1 on fibroblast cells (*8*). Thrombin−PN1 complexes bound to sLRP2 were not competed by RAP although those bound to sLRP3 and sLRP4 were. This result is consistent with our previous observation that RAP only competes for about 65% of the internalization of thrombin−PN1 complexes by astrocyte cells (*47*). In light of the results on apoE and lactoferrin, the finding that binding of sLRP2 to thrombin−PN1 complexes is not RAP inhibitable is less surprising. It is interesting that although the sLRPs are highly homologous, the RAP binding site on sLRP3 and sLRP4 overlaps with the thrombin−PN1 binding site, while on sLRP2 it does not. Future work will be required to localize each of the ligand-binding sites on the sLRPs.

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